# Resistin induces multidrug resistance in myeloma by inhibiting cell death and upregulating ABC transporter expression

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**Supplementary Methods**

*Cell lines, primary myeloma cells, and reagents*

This study was approved by the institutional review board of The University of Texas MD Anderson Cancer Center (Houston, TX). ARP-1 and ARK cells were kindly provided by Arkansas Cancer Research Center, AR. Others were purchased from American Type Culture Collection (ATCC). Primary myeloma cells were isolated from bone marrow aspirates of myeloma patients using anti-CD138 antibody-coated magnetic beads (Miltenyi Biotec, Inc.). Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum and maintained at 37°C with 5% $CO_2$. Recombinant human resistin was purchased from PeproTech. Except where specified, all chemicals were purchased from Sigma-Aldrich, all antibodies for flow cytometry analysis were purchased from BD Biosciences, and all antibodies for Western blot analysis were purchased from Cell Signaling Technology. The siRNAs against human *ABCC5* and *ABCG2* genes as well as the non-target control siRNA were purchased from Santa Cruz Technologies.

*Flow cytometry analysis of cell apoptosis*

Cells were incubated with melphalan or bortezomib, drugs used widely in the treatment of myeloma, with or without resistin, for 24 hours. In some experiments, cells were electroporated first with siRNAs, and then cultured for 48 hours before the drug treatment. Apoptosis of treated cells ($5 \times 10^5$ cells/ sample) was detected by annexin V–FITC/propidium iodide (PI) staining (Life Technologies). After 20 minutes of incubation at room temperature, cells were analyzed by a BD LSRFortessa flow cytometer. Apoptotic cells were defined as the annexin V–positive cells.

*Western blot analysis*

Cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and immunoblotted with antibodies against caspase-9, caspase-3, poly (ADP-ribose) polymerase (PARP), Bcl-2, Bcl-xL, Bax, NF/κB p65, p105/50, p100/52, RelB, Histone H3, phosphorylated (p)IκBα (Ser32/36), IκBα, pp65 (Ser536), pAkt (Ser473), Akt, pERK1/2 (Thr202/Tyr204), ERK1/2, ABCG2, ABCC5, DNMT1, or DNMT3a. GAPDH served as a loading control.

*Quantitative real-time polymerase chain reaction (real-time PCR)*

Total RNA was isolated by using an RNeasy kit (Qiagen). Total RNA (1 μg) was reverse-

transcribed by using a SuperScript II reverse-transcriptase PCR kit (Invitrogen); 1 μL of the final cDNA was used for real-time PCR amplification with SYBRGreen using a StepOnePlus real-time PCR system (Applied Biosciences). Primer sequences are shown in Table 1.

*eFluxx-ID Gold uptake assay for ABC transporter activity*

Fluorescent probe eFluxx-ID Gold was used to monitor activity of ABC transporters (ENZO Life Sciences, Inc.). Briefly, equal number of cells (5 x$10^5$) were added to tubes that contain inhibitors or control solution at 37°C for 5 min. Inhibitors used include 40 μM Verapamil (multidrug resistance 1 inhibitor), 100 μM MK-571 (multidrug resistance-associated protein 1 inhibitor), and 200 μm Novobiocin (Breast cancer resistance protein inhibitor). The fluorescent probe eFluxx-ID Gold were added to the tubes and incubated for 30 minutes at 37°C. To exclude dead cells from the analyses, PI was added to the cells during the last 5 minutes of incubation. The cells were analyzed immediately afterward by flow cytometry. eFluxx-ID Gold fluorescence intensity was measured in the FL2/PE (530/42 nm filter) channel. Analysis of 10,000 cells per sample was carried out in the fluorescence/count four decades log diagram, collecting the autofluorescence signal in the first decade.

*DNA methylation analysis*

Genomic DNA was obtained from treated ARP-1 and MM.1S cells by using the QIAamp Tissue kit (Qiagen) according to the manufacturer's protocol. DNA was processed by bisulfite modification using the Zymo EZ DNA methylation kit (Zymo Research) according to the manufacturer's instructions. Specific primers for methylation-specific PCR (MS-PCR, Table 2) were designed by using MethylPrimer software (http://www.urogene.org/methprimer/index1.html). PCR products were subjected to electrophoresis on a 2% agarose gel.

*In vivo mouse model*

Six- to eight-week-old CB.17 SCID mice were obtained from Charles River Laboratories and maintained in facilities accredited by the American Association of Laboratory Animal Care. The studies were approved by the Institutional Animal Care and Use Committee of MD Anderson Cancer Center. ARP-1 myeloma cells ($5\times10^5$ cells/mouse) were injected into mouse femurs. After 3 weeks, treatment with melphalan (50 μg/mouse) or resistin (50 μg/mouse), singly

or in combination, was begun; each mouse received intraperitoneal treatment every 3 days for 3 weeks. Control mice received equal amounts of phosphate-buffered saline solution (PBS). After treatment, mouse sera were collected and serum M-protein levels were measured by the Human Kappa ELISA kit (Bethyl Laboratories). Bone marrow cells were flushed from mouse femurs and the human $CD138^+$ subset was isolated by using anti-human CD138-coated magnetic beads. Cells were then labeled with antibodies and analyzed with an LSRFortessa flow cytometer (BD Biosciences).

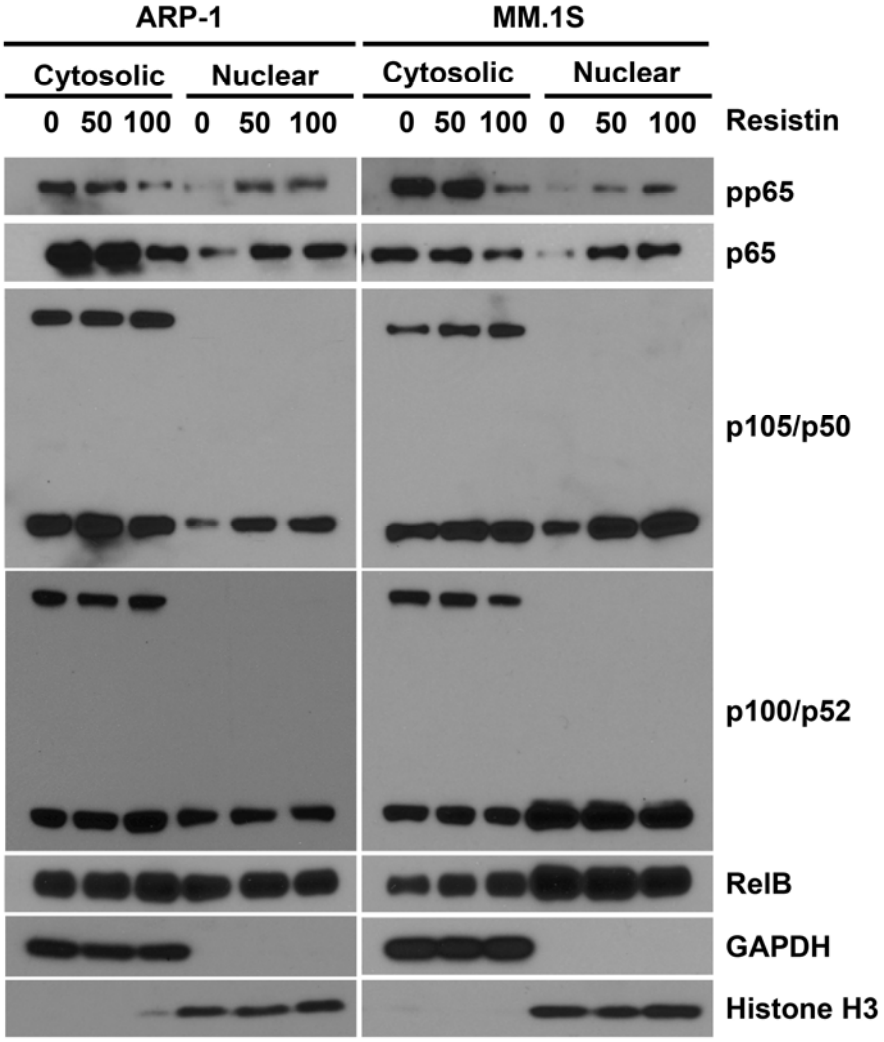
*In situ TUNEL assay*

Mouse femurs were fixed in 10% neutral-buffered formalin for 18 hours and embedded in paraffin. Later, the bones were sectioned and deparaffinized. *In situ* tumor cell apoptosis was determined by a TUNEL assay kit (Roche Life Science). Three sections from each bone specimen were randomly selected and examined using a defined rectangular field area at ×200 magnification.

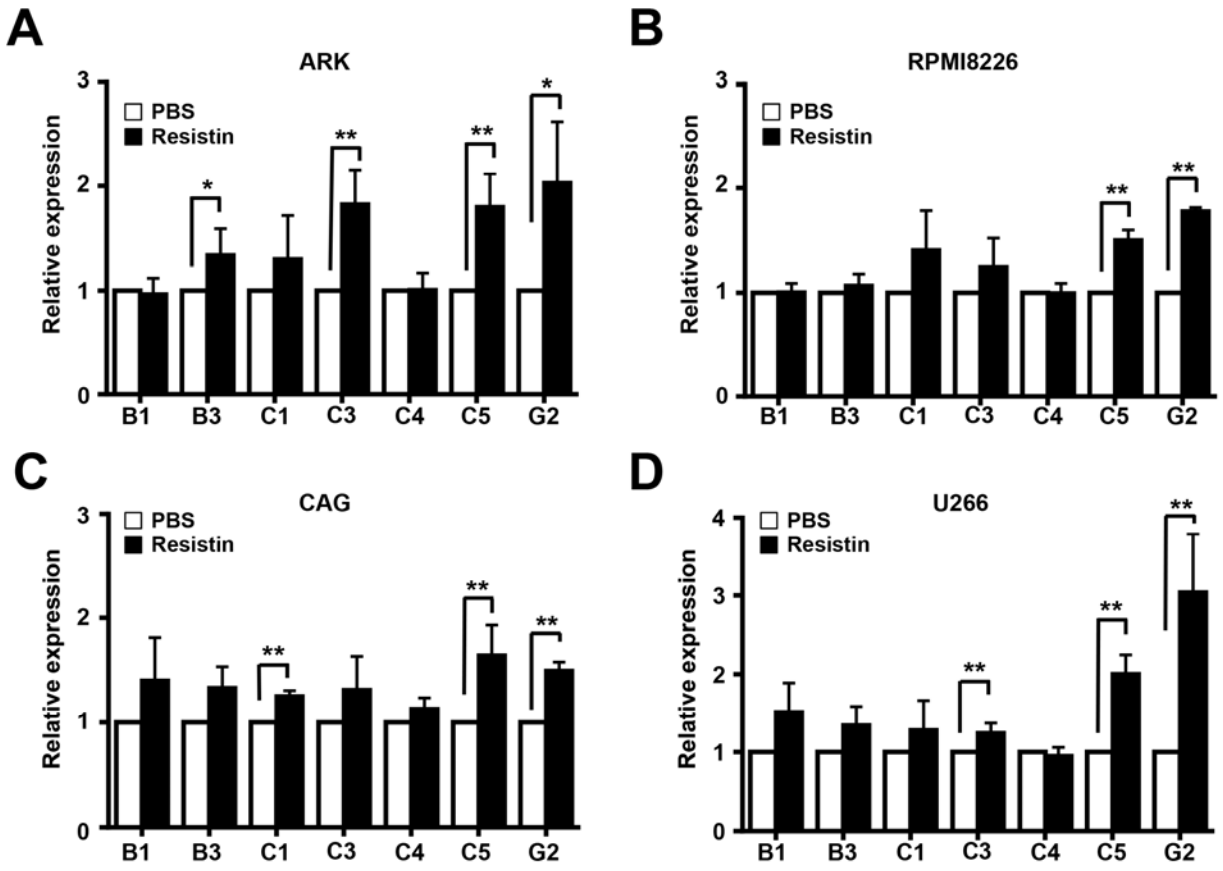
*Statistical analysis*

All data are shown as means ± standard deviation for at least three independent experiments performed in triplicate. The Student *t*-test was used to compare experimental groups. A $P$ value $<0.05$ was considered statistically significant.

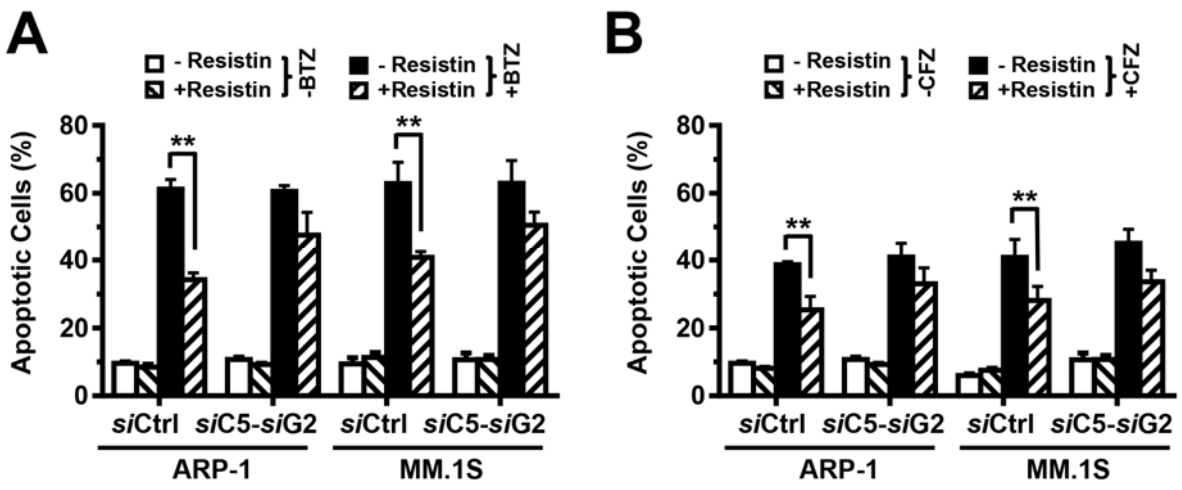
## Supplementary Figure Legend




**Supplementary Figure 1. Resistin activates the NF-κB signaling pathway in myeloma cells.** The myeloma cell lines ARP-1 and MM.1S were cultured in medium with or without resistin (0, 50, or 100 ng/mL) for 12 hours. Western blot analysis shows the levels of phosphorylated (p) p65 and the levels of p65, p105/p50, p100/p52, and RelB in nuclear or cytosolic fraction of the myeloma cells. The levels of GAPDH served as a loading control for cytosolic proteins and the levels of histone H3 served as a loading control for nuclear proteins. Results shown are representative of three independent experiments.




**Supplementary Figure 2. Resistin regulates the expression of ABC transporters in multiple cell lines of myeloma.** The human myeloma cell lines were cultured with resistin (50 ng/mL) for 12 hours. After cultures, the cells were subjected to real-time PCR analysis. Shown is the relative mRNA expression of *ABC* transporter genes in ARK (**A**), RPMI8226 (**B**), CAG (**C**), and U266 (**D**). Results shown are representative of three independent experiments. **P* <0.05; ***P* <0.01.




**Supplementary Figure 3. Knockdown of ABCC5 and ABCG2 abrogates the effect of resistin on inhibition of myeloma cell apoptosis.** The myeloma cell lines ARP-1 and MM.1S were transfected with non-targeted *si*RNAs, served as controls (*si*Ctrl), or pooled *si*RNAs of ABCC5 and ABCG2 (*si*C5-*si*G2). The cells were then cultured without or with resistin (50 ng/mL) or the drug bortezomib (BTZ; 5 nM) or carfilzomib (CFZ; 20 nM) for 12 hours. Shown is the percentage of apoptotic cells in *si*Ctrl- or *si*C5-*si*G2- expressing ARP-1 or MM.1S cells treated with or without resistin, BTZ (**A**), or CFZ (**B**). Results shown are representative of three independent experiments. ***P* <0.01.